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<b>(54) Title:</b> METHOD FOR IDENTIFYING TRANSLATIONALLY REGULATED GENES			
<b>(57) Abstract</b>  A method for identifying translationally regulated genes includes selectively stimulating translation of an unknown target mRNA with a stress inducing element wherein the target mRNA is part of a larger sample of mRNA. The mRNA sample is divided into pools of translated and untranslated mRNA which are differentially analyzed to identify genes that are translationally regulated by the stress inducing element. A method for identifying gene sequences coding for internal ribosome entry sites includes inhibiting 5'cap-dependent mRNA translation in a cell, collecting a pool of mRNA from the cells, and differentially analyzing the pool of mRNA to identify genes with sequences coding for internal ribosome entry sites.			

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## METHOD FOR IDENTIFYING TRANSLATIONALLY REGULATED GENES

### BACKGROUND OF THE INVENTION

#### Technical Field

The present invention relates to a method for identifying genes that are translationally regulated. More specifically, the present invention relates to the rapid isolation of differentially expressed or developmentally regulated gene sequences through segregation of mRNAs into translated and untranslated pools and comparing the relative abundance of the mRNAs found in these pools by differential analysis.

#### Background Art

The identification and/or isolation of genes whose expression differs between two cell or tissue types, or between cells or tissues exposed to stress conditions, chemical compounds or pathogens, is critical to the understanding of mechanisms which underlie various physiological conditions, disorders, or diseases. Regulation of gene expression has been shown to play an important part in many biological processes including embryogenesis, aging, tissue repair, and neoplastic transformation. Gene regulation at the level of translation has been shown to be of critical importance. For example, it has been demonstrated that a group of mRNAs are stored in an egg as a pool of untranslated mRNAs which, following fertilization, shift into the pool of translated mRNAs. Another example of a change in the translational state of mRNA is a subgroup of mRNAs which code for heat shock proteins which are not translated under normal physiological conditions. These mRNAs begin to be translated following exposure of cells to high temperatures.

A number of methods have been developed for the detection and isolation of genes which are activated or repressed in response to developmental, physiological, pharmacological, or other cued events. One particular method is described in United States Patent Number 5,525,471 to Zeng, is subtractive

hybridization. Subtractive hybridization is a particularly useful method for selectively cloning sequences present in one DNA or RNA population but absent in another. The selective cloning is accomplished by generating single stranded complementary DNA libraries from both control cells/tissue (driver cDNA) and cell/tissue during or after a specific change or response being studied (tester cDNA). The two cDNA libraries are denatured and hybridized to each other resulting in duplex formation between the driver and tester cDNA strands. In this method, common sequences are removed and the remaining non-hybridized single-stranded DNA is enriched for sequences present in the experimental cell/tissue which is related to the particular change or event being studied. (Davis et al., 1987).

Currently used methodologies to identify mRNAs encoding proteins which are being induced/reduced following a cue or stimulus rely on changes in the mRNA levels following transcriptional induction/repression via screening of differentially expressed mRNAs. One such method for the identification of differentially expressed mRNAs is disclosed in United States Patent Number 5,459,037 to Sutcliffe et al. According to this method, an mRNA population is isolated, double-stranded cDNAs are prepared from the mRNA population using a mixture of twelve anchor primers, the cDNAs are cleaved with two restriction endonucleases, and then inserted into a vector in such an orientation that they are anti-sense with respect to a T3 promotor within the vector. *E. coli* are transformed with the cDNA containing vectors, linearized fragments are generated from the cloned inserts by digestion with at least one restriction endonuclease that is different from the first and second restriction endonucleases and a cDNA preparation of the anti-sense cDNA transcripts is generated by incubating the linearized fragments with a T3 RNA polymerase. The cDNA population is divided into subpools and the first strand cDNA from each subpool is transcribed using a thermostable reverse transcriptase and one of sixteen primers. The transcription product of each of the sixteen reaction pools is used as a template for a polymerase chain reaction (PCR) with a 3'-primer and a 5'-primer and the polymerase chain reaction amplified fragments are resolved by electrophoresis to display bands

representing the 3'-ends of the mRNAs present in the sample. This method is useful for the identification of differentially expressed mRNAs and the measurement of their relative concentrations. This type of methodology, however, is unable to identify mRNAs whose levels remain constant but their translatability is variable or changes.

Schena et al. developed a high capacity system to monitor the expression of many genes in parallel utilizing microarrays. The microarrays are prepared by high speed robotic printing of cDNAs on glass providing quantitative expression measurements of the corresponding genes (Schena et al., 1995). Differential expression measurements of genes are made by means of simultaneous, two color fluorescence hybridization. However, this method alone is insufficient for the identification of translationally regulated genes.

The use of a known inhibitor of hypusine formation, mimosine, was used to reversibly suppress the hypusine-forming deoxyhypusyl hydroxylase in cells while differentially displaying their polysomal versus non-polysomal mRNA populations. (Hanauske-Abel et al., 1995) Utilizing this method, several species of mRNA were discovered which disappear and reappear, respectively, at polysomes in connection with inhibition and disinhibition of hypusine formation and which are thought to code for translationally controlled enzymes. This method only teaches the use of a known stimulating element (i.e., inducer or repressor) to identify translationally regulated genes. This method does not provide a mechanism for the detection and/or identification of translationally regulated genes where the stimulating element is unknown.

Generally, the translation of eukaryotic mRNAs is dependent upon 5' cap-mediated ribosome binding. Prior to translation, the ribosome small sub-unit (40S) binds to the 5'-cap structure on a transcript and then proceeds to scan along the mRNA molecule to the translation initiation site where the large sub-unit (60S) forms the complete ribosome initiation site. In most instances, the translation initiation site is the first AUG codon. This "scanning model" of translation initiation accommodates most eukaryotic mRNAs. A few notable exceptions to the "scanning model" are provided by the Picornavirus family.

These viruses produce non-capped transcripts with long (600-1200 nucleotides) 5'-untranslated regions (UTR) which contain multiple non-translation initiating AUG codons. Because of the absence of a cap structure, the translational efficiency of these RNAs is dependent upon the presence of specific sequences within the untranslated regions (UTR) known as internal ribosome entry sites (IRES).

More recently, IRES containing mRNA transcripts have been discovered in non-viral systems such as the mRNA encoding for immunoglobulin heavy chain binding protein, the *antenapedia* gene in *Drosophila*, and the mouse *Fgl-2* gene. These discoveries have promoted speculation for the role of cap-independent translation in the developmental regulation of gene expression during both normal and abnormal processes.

The discovery of the above-mentioned non-viral IRES containing mRNAs implies that eukaryotic IRES sequences could be more wide spread than has been previously realized. The difficulty in identifying eukaryotic IRES sequences resides in the fact that they typically cannot be identified by sequence homology. [Oh et al., 1993; Mountford et al., 1995; Macejak et al., 1991; Pelletier et al., 1988; Vagner et al. 1995] It would, therefore, be advantageous to have a method for identifying IRES containing mRNA in order to identify translationally controlled genes operating via 5'-cap independent translation in order to ascertain and assess their association with both normal and abnormal processes.

Therefore, it would be desirable to have a rapid, reliable, and reproducible method for the identification and cloning of clinically and therapeutically relevant differentially expressed genes which will overcome the inherent problems associated with the prior art methods.

## SUMMARY OF THE INVENTION

According to the present invention, there is provided a method for identifying translationally regulated genes in an organism including the steps of selectively stimulating translation of an unknown target mRNA with a stress inducing element, the target mRNA being part of a larger sample of mRNA, dividing the sample of mRNA into pools of translated and untranslated mRNA and

differentially analyzing the pools of mRNA to identify genes translationally regulated by the stress inducing element. The stress inducing element can include pathologic, environmental including chemical and physical stressors or other stimulus that induces mRNA translation. Also, in accordance with the present invention, there is provided a method for identifying gene sequences coding for internal ribosome entry sites. The method includes inhibiting 5'cap-dependant mRNA translation in a cell, collecting a pool of mRNA from the cells, and differentially analyzing the pool of mRNA to identify genes with sequences coding for internal ribosome entry sites.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1A is an absorbance profile of a fractionation of cytoplasmic RNA on a sucrose density gradient wherein the absorbance (at 254nm) is plotted against the sedimentation rate of the cytoplasmic RNA;

Figure 1B is a photograph of purified RNA electrophoresed on an agarous gel and stained with ethidium bromide illustrating the fractionation of RNA;

Figure 2 is a photograph of a 5% acrylamide gel illustrating a differential translation analysis of mRNA from sucrose density gradients according to the present invention;

Figure 3A-C are schematic representations of plasmids that contain the Polio virus 2A genes (A) in plasmid pTK-OP3-WT2A, (B) in the plasmid miniTK-WT2A, and (C) in a plasmid containing a hygromycin selectable marker;

Figure 4 is graph illustrating the induction of Polio virus 2A protease leading to cell death after induction of the 2A protease;

Figure 5 is a photograph of a gel illustrating the presence of Polio virus 2A protease expression in transformed HEK-293 cells (293-2A) following

induction with IPTG and the absence of the Polio virus 2A protease in HEK-293 (293) parental cells following treatment with IPTG; and

Figure 6 is a photograph of a Western blot illustrating the activity of the Polio virus 2A protease in cleaving the p220 protein component of the 40S ribosomal subunit demonstrating that clones which were induced for Polio virus 2A protease generated cleavage products of the p220 protein.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for identifying translationally regulated genes in an organism by selectively stimulating translation of an unknown target mRNA with a stress inducing element, the target mRNA being part of a larger sample. The organism may be any organism which provides suitable mRNA. The mRNA sample is divided into pools of translated and untranslated mRNA which are differentially analyzed to identify genes which are translationally regulated by the stress inducing element. This method is designed for identifying and cloning genes which are regulated at the translational level. That is, the present method is designed for identifying and cloning genes which are either up- or down- regulated including identifying genes responsive to a specific pathology or stress condition.

The method of the present invention provides a novel approach to the identification and cloning of genes that are involved in fundamental cellular functions and which are regulated at the level of translation in an organism. The basic underlying theory for this method relies on the assumption that an mRNA encoding a protein required for a quick response to an external cue is generally stored as an untranslated mRNA. Following the appropriate external cue, the mRNA is translated and the encoded protein quickly appears. By comparing mRNA populations that are "active" or "non-active" at a given time, genes that are regulated by a mechanism referred to as the "shift mechanism" can be identified.

The method can also be applied to identify in addition to genes regulated at the translational level; genes regulated at the transcription level; genes regulated by RNA stability; gene regulated by mRNA transport rate between the



nucleus and the cytoplasm; and gene regulated by differential splicing. That is, genes whose expression in part, is controlled/regulated at the mRNA level can be identified.

5 The method will identify genes encoding secreted and membrane proteins; genes encoding for nuclear proteins; genes encoding for mitochondrial proteins; and genes encoding for cytoskeletal proteins. In addition, any other gene whose expression can be controlled at the mRNA level can be identified by this method.

10 As used herein, RNA refers to RNA isolated from cell cultures, cultured tissues or cells or tissues isolated from organisms which are stimulated, differentiated, exposed to a chemical compound, are infected with a pathogen or otherwise stimulated. As used herein, translation is defined as the synthesis of protein on an mRNA template.

15 As used herein, the term stimulating translation of unknown target mRNA or stimulating element includes chemically, pathogenically, physically, or otherwise inducing or repressing an mRNA population from genes which can be derived from native tissues and/or cells under pathological and/or stress conditions that are regulated by the "shift mechanism." In other words, stimulating the translation of mRNA with a stress inducing element or "stressor" can include the application of an external cue, stimulus, or stimuli which stimulates or initiates translation of a mRNA stored as untranslated mRNA in the cells from the sample. In addition to stimulating translation of mRNA from genes in native cells/tissues, stimulation can include induction and/or repression of genes under pathological and/or stress conditions. The present method utilizes a stimulus or stressor to identify unknown target genes which are translationally regulated by the stress inducing element or stressor.

25 The method of the present invention integrates two previously known methodologies which were otherwise used separately. The first method is the division of an mRNA sample into separate translated and untranslated pools of mRNA. The second methodology involves the simultaneous comparison of the relative abundance of the mRNA species found in the separate pools by a method

of differential analysis such as differential display, representational difference analysis (RDA), gene expression microarray (GEM), suppressive subtraction hybridization (SSH) (Diatchenko et al., 1996), and techniques such as chip technology exemplified by United States Patent No. 5,545,531 to Rava et al.  
5 assigned to Affymax Technologies N.V. and direct sequencing exemplified by WO 96/17957 patent application to Hyseq, Inc.

Briefly, subtractive hybridization is defined as subtraction of mRNA by hybridization in solution. RNA that are common to the two pools form a duplex that can be removed, enriching for RNAs that are unique or more  
10 abundant in one pool. Differential Display is defined as reverse transcription of mRNA into cDNA and PCR amplification with degenerated primers. Comparison of the amounts amplification products (by electrophoresis) from two pools indicate transcript abundance. RDA, GEM, SSH, SAGE are described herein above.

The specific cells/tissues which are to be analyzed in order to  
15 identify translationally regulated genes, can include any suitable cells and/or tissues. Any cell type or tissue can be used, whether an established cell line or culture or whether directly isolated from an exposed organism.

The cells/tissues to be analyzed under the present method are selectively stimulated utilizing a physiological, chemical, environmental and/or  
20 pathological stress inducing element or stressor, in order to stimulate the translation of mRNA within the sample tissue and identify genes whose expression is regulated at least in part at the mRNA level. Following the stimulation of the translation of RNA, the RNA from the cells/tissues is isolated or extracted from the cells/tissues. The isolation of the RNA can be performed utilizing techniques  
25 which are well known to those skilled in the art and are described, for example, in "Molecular Cloning: A Laboratory Manual" (Cold Springs Harbor Laboratory Press, Cold Spring Harbor, New York, 1989). Other methods for the isolation and extraction of RNA from cells/tissue can be used and will be known to those of ordinary skill in the art. (Mach et al., 1986, Jefferies et al., 1994).

30 Following the isolation of the pool of translated and untranslated mRNA, the mRNAs which are actively engaged in translation and those which

remain untranslated can be separated utilizing a procedure such as fractionation on a sucrose density gradient, high performance gel filtration chromatography, or polyacrylamide gel matrix separation (Ogishima et al., 1984, Menaker et al., 1974, Hiramata et al., 1986, Mechler, 1987, and Bharucha and Murthy, 1992), since  
5 mRNAs that are being translated are loaded with ribosomes and, therefore, will migrate differently on a density gradient than ribosome-free untranslated mRNAs. By comparing mRNA populations that are active or non-active in translation at a given time, genes that are regulated by the "shift mechanism" can be identified.

Polysomal fractionation and specific analysis can be facilitated by  
10 treatment of target cell/tissue with drugs that will specifically inhibit or modulate transcription or translation. Examples of such drugs are actinomycin D and cyclohexamide, respectively.

The fractionation can be completed to create polysomal subdivisions. The subdivisions can be made to discriminate between total  
15 polyribosomes or membrane bound ribosomes by methods known in the art (Mechler, 1987). Further, the mRNA sample can be in addition fractionated into one or more of at least the following subsegments or fractions: cytoplasmatic, nuclear, polyribosomal, sub polyribosomal, microsomal or rough endoplasmic reticulum, mitochondrial and spliceosome associated mRNA by methods known in  
20 the art (see also Table 1).

Following isolation and division of the total mRNA population into separate translated and untranslated pools of mRNA, the relative abundance of the many mRNA species found in these pools are simultaneously compared using a differential analysis technique such as differential display, representational  
25 difference analysis (RDA), GEM-Gene Expression Microarrays (Schena et al., 1995, Aiello et al., 1994, Shen et al., 1995, Bauer et al., 1993, Liang and Pardee, 1992, Liang and Pardee, 1995, Liang et al., 1993, Braun et al., 1995, Hubank and Schatz, 1994) and suppressive subtraction hybridization (SSH). The RNA isolated from the fractions can be further purified into mRNA without the ribosomal RNA  
30 by poly A selection. It should be noted that multiple pools can be analyzed

utilizing this method. That is, different cell aliquots subjected to different stressors can be compared with each other as well as with the reference sample.

Labeled mRNA (in a cDNA or PCR product form) from polysomal, non-polysomal or mRNPs (pools or individual fractions) can be used as probes, to identify clones of cDNA, genomic clones, and mRNA species that are fixed onto a solid matrix-like microarrays such as (GEM), that shown in United States Patent Number 5,545,531 to Rava et al. and WO96/17957 to Hyseq, Inc., and membranes of any kind where clones can be either blotted after electrophoresis or directly loaded (dot blot) onto the membrane. The label can be radioactive, fluorescent, or incorporating a modified base such as digoxigenin and biotin.

Comparison between the fractions derived from the polysomal or polyribosomal fraction or other fractions to the total unfractionated material is essential to discriminate between differentials in expression levels that are the result of transcription modulation from those that result from modulation of translation per se. The polysomal fractions or groups can include membrane bound polysomes, loose or tight polysomes, or free unbound polysome groups.

The importance of utilizing the polysomal sub-population in order to identify differentially (translationally) expressed genes is shown in Example 2 where a number of genes were not detected as translationally expressed under heat shock inducement when total mRNA was used as the detection probe but, however, when polysomal mRNA was used as a probe, a number of genes were identified as differentially expressed. These genes were previously thought to be non-differentially expressed when total mRNA was used as a probe. That is, as shown in Example 2, a number of genes that were not detected as translationally expressed under heat shock inducement with total mRNA were detected when probed with polysomal mRNA fractions.

The present method for identifying translationally regulated genes is not limited by the source of the mRNA pools. Therefore, the present method can be utilized to clone genes from native cells/tissue under pathological and/or stress conditions that are regulated by the "shift mechanism," as well as genes that are induced/repressed under pathological and/or stress conditions. Pathologies can

include disease states including those diseases caused by pathogens and trauma. Stress conditions can also include disease states, physical and psychological trauma, and environmental stresses. Following analysis by the selected method of differential analysis, the genes which have been identified as being regulated by translation can be cloned by any suitable cloning methodologies known to those skilled in the art. (Lisitsyn and Wigler, 1993).

Differential comparisons can be made of all possible permutations of polysomal vs. non-polysomal RNA where the definition of the fraction type is done, for example, by absorbance profile at 254nm, density of the sucrose gradient as shown in Figure 1A (or another size standard if high pressure liquid chromatography or gel systems are used) and types of RNA that are stained with ethidium bromide after electrophoresis of the fractions on agarous gels are completed, as shown in Figure 1B. In Figure 1A, the polysomal fractions are those that have mRNA with more than two ribosomes loaded. The materials and methods for this comparison are set forth below in the experimental section.

Differential comparisons can also include polysomal vs. non-polysomal fractions in each condition. By "condition" it is meant that cells from the same source, such as a cell line, a primary cell, or a tissue that undergoes different treatment or has been modified to have different features or to express different sets of genes. For example, this can be accomplished by differentiation, transformation, application of the stress such as oxygen deprivation, chemical treatment, or radiation. Permutations can include, for example:

1. polysomal fractions between conditions individually (migrating in the same density) or in a pool;
2. non-polysomal fractions between conditions individually (migrating in the same density) or in a pool;
3. non-polysomal to polysomal between conditions and within each condition individually (migrating in the same density) or in a pool; and
4. each of the fractions being polysomal and non-polysomal individually (migrating in the same density) or in a pool that can be compared to total RNA that is unfractionated.

The method described above for the identification of translationally regulated genes has a number of applications. A particular application for this method is its use for the detection of changes in the pattern of mRNA expression in cells/tissue associated with any physiological or pathological change. By  
5 comparing the translated versus untranslated mRNAs, the effect of the physiological or pathological cue or stress on the change of the pattern of mRNA expression in the cell/tissue can be observed and/or detected. This method can be used to study the effects of a number of cues, stimuli, or stressors to ascertain their effect or contribution to various physiological and pathological activities of the  
10 cell/tissue. In particular, the present method can be used to analyze the results of the administrations of pharmaceuticals (drugs) or other chemicals to an individual by comparing the mRNA pattern of a tissue before and after the administration of the drug or chemical. This analysis allows for the identification of drugs, chemicals, or other stimuli which affect cells/tissue at the level of translational  
15 regulation. Utilizing this method, it is possible to ascertain if particular mRNA species are involved in particular physiological or disease states and, in particular, to ascertain the specific cells/tissue wherein the external stimulus, i.e., a drug, affects a gene which is regulated at the translational level.

A further embodiment of the present invention provides a method  
20 for identifying gene sequences coding for internal ribosome entry sites (IRES) and includes the general steps of inhibiting 5'-cap-dependant mRNA translation in a cell, collecting a pool of mRNA from the cells, and differentially analyzing the pool of mRNA to identify genes with sequences coding for internal ribosome entry sites.

25 As described above, it is known that an exception to the standard 5'-cap dependent translation initiation exists. Sequences exist within untranslated regions (UTRs) of RNAs which can include the presence of specific sequences known as internal ribosome entry sites (IRES). (Ehrenfeld, 1996) These internal ribosome entry sites have been shown to support translation initiation for several  
30 prokaryotic and eukaryotic systems as set forth above. However, in order to identify translationally controlled genes via 5'-cap independent translation

mechanisms and their association with both normal and abnormal processes, it is necessary to inhibit 5'-cap initiated translation so that 5'-cap independent mRNA translation can be selected for. This inhibition is necessary since IRES sequences are difficult, if not impossible, to identify by sequence homology.

5                   In order to inhibit 5'-cap dependent translation and thereby select for the presence of 5'-cap independent translation, cells or tissues which are to be analyzed for the presence of internal ribosome entry sites must be treated in some manner to prevent or discourage the 5'-cap translation initiation mechanism. The mechanism(s) of standard scanning-type translation initiation should be  
10 substantially, if not totally, turned off or shut down to, in essence, shift the translation equilibrium in favor of IRES initiated translation. That is, recognition of the 5'-cap structure is inhibited by disrupting the normal mechanism for 5'-cap mediated initiation. The mechanism for inhibiting the 5'-cap translation can include any known means or mechanisms for preventing the initiation of 5'-cap  
15 mediated translation. One such mechanism for inhibiting 5'-cap mediated translation is the expression of Polio virus 2A protease into a cell, cell system, or tissue to be analyzed for the presence of IRES sequences. The use of the Polio virus 2A protease inhibits 5'-cap-dependent mRNA translation by inactivating the cellular 5'-cap-dependent translation machinery. This enables the identification of  
20 cellular IRES containing genes which may be translationally controlled and play a critical role in the immediate response of the cell following the application of a stress inducing element/stressor such as heat shock, hypoxia, or other stress inducing elements as set forth above, prior to gene activation. The Polio virus 2A protease prevents 5'-cap-mediated translation by cleaving the large sub-unit of eIF-  
25 4 $\gamma$  (p220) of eukaryotic translation initiation factor 4 (eIF-4) which is involved in the recognition of the mRNA 5'-cap.

                  In order to inhibit the 5'-cap-mediated translation, the Polio virus 2A protease must be incorporated into the cell or cells being analyzed for the presence of gene sequences coding for internal ribosome entry sites and/or for  
30 identifying translationally regulated genes. One such method for incorporating the Polio virus 2A protease into a cell involves the transformation of a target cell with

an expression vector containing the gene which codes for the Polio virus 2A protease. Because the Polio virus 2A protease is deleterious to living cells when it is constitutively expressed, the expression vector containing the Polio virus 2A protease gene is coupled with a bacterial LacI inducible system wherein a LacI repressor is constitutively expressed under a CMV promoter. The Polio virus 2A protease may be expressed under a number of suitable promoters including the RSV, the TK, or the mini-TK promoter coupled at their 3' end to the LacI repressor binding sites. By transforming the target cells with an expression vector containing the LacI repressor and the Polio virus 2A expression vector, the expression of the Polio virus 2A protease can be induced upon treatment of the cells with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Treatment of the target cells with IPTG relieves the binding of the LacI repressor molecules bound at the repressor binding sites thus enabling transcription of the Polio virus 2A protease. By coupling the expression of the Polio virus 2A protease to an inducible system, such as the LacI system, this mechanism allows for the establishment of control of the expression of the gene coding for the Polio virus 2A protease.

Examples of an embodiment of the present invention for identifying gene sequences coating for internal ribosome entry sites are set forth below in the examples.

Following induction of the expression of the Polio virus 2A protease in the target cells, RNA, presumably containing internal ribosome entry sites, can be collected and analyzed utilizing the methods described above to identify genes whose translation is up-regulated by the effects of the Polio virus 2A protease.

## EXPERIMENTAL

### DIFFERENTIAL TRANSLATION

#### MATERIALS AND METHODS

##### General Scheme

- a. Total mRNA organic extraction of all RNA from the source tissue or cell. (additional selection for polyA+ mRNA can be included).



- b. Nuclear RNA-lysis of cells (from a tissue or a cell line) by homogenization in hypotonic buffer. Collection of nuclei by centrifugation and organic extraction of the RNA.
- 5 c. Organic extraction of the RNA from the supernatant from 2 above.
- d. Polyribosomal/subpolyribosomal fractionation. Lysis of cells by homogenization hypotonic buffer, removal of nuclei and fractionation of polyribosome on linear sucrose gradients and organic extraction of the RNA from each fraction of the gradient.
- 10 e. Secreted and membrane encoding transcripts.
1. Isolation of RER on Percol gradients (after homogenization of cells).
  2. Preparation of microsomes containing the RER
  3. Isolation of membrane-bound polyribosomes by successive treatment of cells with detergents.
- 15 f. Nuclear proteins. Isolation of cytoskeletal associated polyribosomes by treating cells lysates with different detergents.
- g. Mitochondrial genes. Isolation of mitochondria on Percoll gradients.
- i. Alternative splicing. Separation of nuclei and isolation of splicosome (proteins and RNA complex) on linear sucrose gradients.

20

#### Preparation of cell extracts

Cells were centrifuged. The pellet was washed with PBS and recentrifuged. The cells were resuspended in 4x of one packed cell volume (PCV) with hypotonic lysis buffer (HLB: 20mM TrisHCL pH=7.4; 10mM NaCl; 3mM MgCl<sub>2</sub>). The

25 cells were incubated five minutes on ice. 1xPCV of HLB containing 1.2% Triton X-100 and 0.2M sucrose was added. The cells were homogenized with a Dounce homogenizer (five strokes with B pestle). The cell lysate was centrifuged at 2300g for ten minutes at 4°C. The supernatant was transferred to a new tube. HLB containing 10mg/ml heparin was added to a final concentration of 1mg/ml heparin.

30 NaCl was added to a final concentration of 0.15M. The supernatant was frozen at -70°C after quick freezing in liquid N<sub>2</sub> or used immediately.

### Sucrose gradient fractionation

A linear sucrose gradient from 0.5M to 1.5M sucrose in HLB was prepared.

5 Polyallomer tubes (14X89mm) were used. 0.5 to 1.0ml of cell extract was loaded on the gradient. The cells were centrifuged at 36,000 RPM for 110 minutes at 4°C. An ISCO Density Fractionator was used to collect the fractions and record the absorbance profile.

### RNA purification

10 SDS was added to 0.5% and Proteinase K to 0.1mg/ml and incubated at 37°C for 30 minutes. Extract with an equal volume of phenol+chloroform (1:1). The aqueous phase was extracted with one volume of chloroform and the RNA was precipitated by adding Na-Acetate to 0.3M and 2.5 volumes of ethanol and incubating at -20°C overnight. Centrifuged ten minutes, the supernatant was  
15 aspirated and the RNA pellet was dissolved in sterile, diethylpyrocarbonate (hereinafter referred to as "DEPC") DEPC-treated water.

### DIFFERENTIAL ANALYSIS

#### Differential display:

20 Reverse transcription: 2µg of RNA were annealed with 1pmol of oligo dT primer (dT)<sub>18</sub> in a volume of 6.5µl by heating to 70°C for five minutes and cooling on ice. 2µl reaction buffer (x5), 1µl of 10mM dNTP mix, and 0.5µl of SuperScript II reverse transcriptase (GibcoBRL) was added. The reaction was carried out for one hour at 42°C. The reaction was stopped by adding 70µl TE (10mM Tris pH=8;  
25 0.1mM EDTA). Oligonucleotides used for Differential display: The oligonucleotides were essentially those described in the Delta RNA Fingerprinting kit (Clontech Labs. Inc.). There were 9 "T" oligonucleotides of the structure: 5' CATTATGCTGAGTGATATCTTTTTTTTTTXY 3' (SEQ ID No: 1). The 10 "P" oligonucleotides were of the structure: 3' ATTAACCCTCACTAAA  
30 "TGCTGGGGA" 3' (SEQ ID No: 11) where the 9 or 10 nucleotides between the

parenthesis represent an arbitrary sequence and there are 10 different sequences (SEQ ID Nos. 12-21), one for each "P" oligo.

Amplification reactions: each reaction is done in 20 $\mu$ l and contains 50 $\mu$ M dNTP mix, 1 $\mu$ M from each primer, 1x polymerase buffer, 1 unit expand Polymerase (Boehringer Mannheim), 2 $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dATP and 1 $\mu$ l cDNA template. Cycling conditions were: three minutes at 95°C, then three cycles of two minutes at 94°C, five minutes at 40°C, five minutes at 68°C. This was followed by 27 cycles of one minute at 94°C, two minutes at 60°C, two minutes at 68°C. Reactions were terminated by a seven minute incubation at 68°C and addition of 20 $\mu$ l sequencing stop solution (95% formamide, 10mM NaOH, 0.025% bromophenol blue, 0.025% xylene cyanol).

Gel analysis: 3-4 $\mu$ l were loaded onto a 5% sequencing polyacrylamide gel and samples were electrophoresed at 2000 volts/40 milliamperes until the slow dye (xylene cyanol) was about 2 cm from the bottom. The gel was transferred to a filter paper, dried under vacuum and exposed to x-ray film.

Recovery of differential bands: bands showing any a differential between the various pools were excised out of the dried gel and placed in a microcentrifuge tube. 50 $\mu$ l of sterile H<sub>2</sub>O were added and the tubes heated to 100°C for five minutes. 1 $\mu$ l was added to a 49 $\mu$ l PCR reaction using the same primers used for the differential display and the samples were amplified for 30 cycles of: one minute at 94°C, one minute at 60°C and one minute at 68°C. 10 $\mu$ l was analyzed on agarous gel to visualize and confirm successful amplification.

#### REPRESENTATIONAL DIFFERENCE ANALYSIS

Reverse transcription: as above but with 2 $\mu$ g polyA<sup>+</sup> selected mRNA.

Preparation of double stranded cDNA: cDNA from previous step was treated with alkali to remove the mRNA, precipitated and dissolved in 20 $\mu$ l H<sub>2</sub>O. 5 $\mu$ l buffer,

2μl 10mM dATP, H<sub>2</sub>O to 48μl and 2μl terminal deoxynucleotide transferase (TdT) were added. The reaction was incubated 2-4 hours at 37°C. 5μl oligo dT (1μg/μl) was added and incubated at 60°C for 5 minutes. 5μl 200 mM DTT, 10 μl 10x section buffer (100mM Mg Cl<sub>2</sub>, 900 mM Hepes, pH 6.6) 16 μl dNTPs (1 mM), and 16 U of Klenow were added and the mixture was incubated overnight at room temperature to generate ds cDNA. 100μl TE was added and extracted with phenol/chloroform. The DNA was precipitated and dissolved in 50μl H<sub>2</sub>O.

Generation of representations: cDNA with DpnII was digested by adding 3μl DpnII reaction buffer 20 V and DpnII to 25μl cDNA and incubated five hours at 37°C. 50μl TE was added and extracted with phenol/chloroform. cDNA was precipitated and dissolved to a concentration of 10ng/μl.

The following oligonucleotides are used in this procedure:

R-Bg1-12 5' GATCTGCGGTGA 3' (SEQ ID No: 22)

R-Bg1-24 5' AGCACTCTCCAGCCTCTCACCGCA 3' (SEQ ID No:23)

J-Bg1-12 5' GATCTGTTCATG 3' (SEQ ID No: 24)

J-Bg1-24 5' ACCGACGTCGACTATCCATGAACA 3' (SEQ ID No:25)

N-Bg1-12 5' GATCTTCCCTCG 3' (SEQ ID No:26)

N-Bg1-24 5' AGGCAACTGTGCTATCCGAGGGAA 3' (SEQ IDNo:27)

R-Bg1-12 and R-Bg1-24 oligos were ligated to Tester and Driver: 1.2μg DpnII digested cDNA. 4μl from each oligo and 5μl ligation buffer X10 and annealed at 60°C for ten minutes. 2μl ligase was added and incubated overnight at 16°C. The ligation mixture was diluted by adding 140μl TE. Amplification was carried out in a volume of 200μl using R-Bg1-24 primer and 2μl ligation product and repeated in twenty tubes for each sample. Before adding Taq DNA polymerase, the tubes were heated to 72°C for three minutes. PCR conditions were as follows: five minutes at 72°C, twenty cycles of one minute at 95°C and three minutes at 72°C, followed by ten minutes at 72°C.

Every four reactions were combined, extracted with phenol/chloroform and precipitated. Amplified DNA was dissolved to a concentration of 0.5μg/μl and all samples were pooled.

Subtraction: Tester DNA (20 $\mu$ g) was digested with DpnII as above and separated on a 1.2% agarous gel. The DNA was extracted from the gel and 2 $\mu$ g was ligated to J-Bg1-12 and J-Bg124 oligos as described above for the R-oligos. The ligated Tester DNA was diluted to 10ng/ $\mu$ l with TE. Driver DNA was digested with DpnII and repurified to a final concentration of 0.5 $\mu$ g/ $\mu$ l. Mix 40 $\mu$ g of Driver DNA with 0.4 $\mu$ g of Tester DNA. Extraction was carried out with phenol/chloroform and precipitated using two washes with 70% ethanol, resuspended DNA in 4 $\mu$ l of 30mM EPPS pH=8.0, 3mM EDTA and overlaid with 35 $\mu$ l mineral oil. Denatured at 98°C for five minutes, cool to 67°C and 1 $\mu$ l of 5M NaCl was added to the DNA. Incubated at 67°C for twenty hours. Diluted DNA by adding 400 $\mu$ l TE.

Amplification: Amplification of subtracted DNA in a final volume of 200 $\mu$ l as follows: Buffer, nucleotides and 20 $\mu$ l of the diluted DNA were added, heated to 72°C, and Taq DNA polymerase was added. Incubated at 72°C for five minutes and added J-Bg1-24 oligo. Ten cycles of one minute at 95°C, three minutes at 70°C were performed. Incubated ten minutes at 72°C. The amplification was repeated in four separate tubes. The amplified DNA was extracted with phenol/chloroform, precipitated and all four tubes were combined in 40 $\mu$ l 0.2XTE. Digested with Mung Bean Nuclease as follows: To 20 $\mu$ l DNA 4 $\mu$ l buffer, 14 $\mu$ l H<sub>2</sub>O and 2 $\mu$ l Mung Bean Nuclease (10 units/ $\mu$ l) was added. Incubated at 30°C for thirty-five minutes + First Differential Product (DPI).

Repeat subtraction hybridization and PCR amplification at driver: differential ratio of 1:400 (DPII) and 1:40,000 (DPIII) using N-Bg1 oligonucleotides and J-Bg1 oligonucleotides, respectively. Differential products were cloned into a Bluescript vector at the BAM HI site for analysis of the individual clones.

## EXAMPLE 1

### Differential Translation Analysis of mRNA From Sucrose Density Gradients

C6 glioma cells were grown under normal conditions (Normoxia) or under oxygen deprivation conditions (Hypoxia) for eight hours. The cells were then harvested and cytoplasmic extracts were applied onto sucrose gradients. RNA was extracted from the fractions obtained from the sucrose gradient and pooled into polysomal and non-polysomal samples. Following reverse transcription, the differential display technique was applied using the primers T1 and P10 as set forth in Table 2. The PCR products were separated on a 5% acrylamide sequencing gel. The gel was then dried and exposed to X-ray film. The results are shown in Figure 2 wherein "A" shows an mRNA species apparent only in a non-polysomal fraction of cells after eight hours of hypoxia. This represents a potentially transcriptionally induced mRNA species which was still translationally repressed but which could be actively transcribed after prolonged hypoxia. "B" represents an mRNA species found in the non-polysomal fraction of cells grown under normal oxygen levels which was transferred into the polysomal fraction following hypoxia.

The materials and methods were performed as set forth above. This example demonstrates the utility of the present invention for identifying translationally regulating genes which are regulated by a stress inducing element.

## EXAMPLE 2

### Representative Heat Shock GEM Differential Expression Analysis

#### Materials and Methods

The experimental cells were grown under both normal temperature (37°C) and heat shock temperature (43°C) for four hours. The cells were then harvested and cytoplasmic extracts were obtained and RNA extracted therefrom. Then, the extracted RNA was analyzed utilizing GEM technology as disclosed above.

Tables 3 and 4 demonstrate the utility of utilizing polysomal probes versus total mRNA probes in differential expression analysis to identify genes which are differentially expressed in response to a stimulus such as heat shock.

These Tables illustrate that fibronectin, pyruvate kinase, protein disulfide isomerase, poly(ADPribose) polymerase, thymopoietin, 90Kd heat shock protein, acylamino acid-releasing enzyme,  $\beta$ -spectrin, and pyruvate kinase were all identified as being differentially expressed utilizing a polysomal probe whereas, with the exception of fibronectin, the other proteins were not identified as being differentially expressed when a total mRNA probe was utilized. This example demonstrates the utility of the present invention for identifying translationally or differentially regulated genes which are regulated by a stress inducing element. Additionally, in Table 3, the results of heat shock differential gene expression analysis with both polysomal probes and total mRNA probes is provided. Table 3 illustrates that a number of differentially expressed genes were identified using a polysomal probe whereas when a total mRNA probe was used, these genes were not necessarily identified as being differentially expressed. Table 4 statistically illustrates the number of differentially expressed genes identified utilizing either total mRNA or polysomal mRNA as a probe. Table 4 clearly illustrates that polysomal mRNA probes yielded between two and greater than ten fold increases in the number of differentially expressed genes versus total mRNA probes.

### EXAMPLE 3

#### Identification of IRES Containing Genes

##### Establishment of mammalian cells expressing 2A protease

HEK-293 human (ATCC CRL-1573) cells were used as a model system for Polio virus 2A protease induced expression, since preliminary study indicated that 2A protease enhances expression of IRES containing genes in this cell line. HEK-293 cells were co-transfected with CMV-LacI - (constructed by applicant using techniques known to those skilled in the art) in combination with either one of the Polio virus 2A protease expression vectors PTK-OP3-WT2A, miniTK-WT2A, on PC1bb-LacI-Hyg (constructed by applicant on basis of vectors from Stratagene) as shown in Figures 3A-C, respectively. The LacI expression vector contained a hygromycin selectable marker, and the Polio virus 2A protease expression vector contained a neomycin selectable marker which enabled the

isolation of clones resistant to both markers, presumably expressing both LacI repressor and Polio virus 2A proteins.

Analysis of Polio virus 2A protease expression

5 Death assay: - Resistant clones which grew after selection on hygromycin (50µg/ml) and neomycin (500µg/ml), were treated with IPTG (5mM for 48h + 5mM for further 48h). Cells were then monitored for their viability and the clones that showed full mortality upon Polio virus 2A protease induction, presumably expressing the deleterious effect of the Polio virus 2A protease, were selected for  
10 further analysis. Two such clones were isolated, HEK-293 cells expressing Polio virus 2A protease under the control of a TK promotor (clone #14) and HEK-293 cells expressing the Polio virus 2A protease under the control of a miniTK promoter (clone #1) as shown in Figure 4.

15 Analysis of 2A protease expression: - Direct analysis of the Polio virus 2A protease expression in HEK-293miniTK#1 clones and HEK-293TK#14 clones after IPTG induction was not performed due to the lack of antibodies against the protein. Several currently available techniques can be used to measure changes in gene expression including Northern blot analysis, RNase protection assay, *in situ*  
20 hybridization, and reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR is a very sensitive method, and was used to monitor the induction of the mRNA encoding for Polio virus 2A protease in HEK-293miniTK#1 clones following IPTG treatment. mRNA was prepared from HEK-293 parental cells and HEK-293miniTK-2A clones following treatment with IPTG at different time  
25 points. The RNAs were subjected to the RT-PCR reaction using Polio virus 2A protease specific oligonucleotides:

5'GCAACTACCATTTGGCCACTCAGGAAG3', (SEQ ID No:28) and  
5'GCAACCAACCCTTCTCCACCAGCAG3' and (SEQ ID No: 29).

30 Polio virus 2A protease mRNA was not detected in HEK-293 parental cells, however it was induced following IPTG treatment and reached its highest level after 48 hours of IPTG treatment as shown in Figure 5.



Analysis of 2A protease activity

p220 cleavage: - A well characterized function of Polio virus 2A protease is the cleavage of the p220 protein (4F $\gamma$  translational factor), a component of the 40S ribosomal subunit. Cleavage of p220 yields three N-terminal cleavage products of 100-120KDa molecular weight due to post-translational modification. p220 and its cleavage products were identified by 7% SDS PAGE and Western blot analysis using polyclonal anti-p220 antibodies specifically directed against the N-terminal region p220 as shown in Figure 6. Figure 6 demonstrates such an analysis in which HEK-293 miniTK2A#1 clone and HEK-293TK2A#14 clone were induced for Polio virus 2A protease expression to generate cleavage products of p220. As control, HEK-293 cell lysate was treated with Polio virus 2A protease produced by *in vitro* translation, and was found to generate identical cleavage products with the same mobility on 7% SDS PAGE as in the HEK-293 2A clones.

This system was used as the source of mRNA for polysomal fractionation. RDA analysis was performed using the protocol described above to identify genes whose translation was up-regulated by the effects of the Polio virus 2A protease. Table 5 summarizes the results of analyses performed according to the above-described method and genes isolated thereby.

Throughout this application various publications are referenced by citation and patents by number. Full citations for the publication are listed below. The disclosure of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood the terminology used is intended to be in the nature of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

TABLE 1

## FRACTIONATION

## MEASURES AND IDENTIFIES

RNA associated with:

no fractionation

changes of transcript abundance

Total RNA

Nuclear

Measures denovo synthesis of mRNA

Cytoplasmatic

Changes of transcript abundance

Cytoplasmatic/Nuclear

transport of mRNA from the nucleus to the cytoplasm, increased or decreased stability of mRNA

Nuclear/Cytoplasmatic

Polyribosomal/subpoly  
ribosomal

translationally controlled genes

Rough Endoplasmic Reticulum

differences in the abundance of transcripts encoding membrane and secreted proteins

Microsomes

membrane bound polysomes

Cytoskeletal polyribosomes

differences in abundance of transcript encoding for nuclear proteins

mitochondrial

differences in the abundance of mRNA encoding mitochondrial proteins

Spliceosome

differences in alternative splicing

**TABLE 2**

Primers used in differential Display analysis

T Primers:

5'

- 5 T1: CATTATGCTGAGTGATATCTTTTTTTTAA (SEQ ID No: 2)  
 T2: CATTATGCTGAGTGATATCTTTTTTTTAC (SEQ ID No: 3)  
 T3: CATTATGCTGAGTGATATCTTTTTTTTATAG (SEQ ID No: 4)  
 T4: CATTATGCTGAGTGATATCTTTTTTTTCA (SEQ ID No: 5)  
 T5: CATTATGCTGAGTGATATCTTTTTTTTCC (SEQ ID No: 6)  
 10 T6: CATTATGCTGAGTGATATCTTTTTTTTCG (SEQ ID No: 7)  
 T7: CATTATGCTGAGTGATATCTTTTTTTTGA (SEQ ID No: 8)  
 T8: CATTATGCTGAGTGATATCTTTTTTTTGC (SEQ ID No: 9)  
 T9: CATTATGCTGAGTGATATCTTTTTTTTGG (SEQ ID No: 10)

15 P Primers:

5'

- P1: ATTAACCCTCACTAAATGCTGGGGA (SEQ ID No: 12)  
 P2: ATTAACCCTCACTAAATGCTGGAGG (SEQ ID No: 13)  
 P3: ATTAACCCTCACTAAATGCTGGTAG (SEQ ID No: 14)  
 20 P4: ATTAACCCTCACTAAATGCTGGTAG (SEQ ID No: 15)  
 P5: ATTAACCCTCACTAAAGATCTGACTG (SEQ ID No: 16)  
 P6: ATTAACCCTCACTAAATGCTGGGTG (SEQ ID No: 17)  
 P7: ATTAACCCTCACTAAATGCTGTATG (SEQ ID No: 18)  
 P8: ATTAACCCTCACTAAATGGAGCTGG (SEQ ID No: 19)  
 25 P9: ATTAACCCTCACTAAATGTGGCAGG (SEQ ID No: 20)  
 P10 ATTAACCCTCACTAAATGCACCGTCC (SEQ ID No: 21)

**TABLE 3**

### Heat Shock Differential Gene Expression Analysis with Polysomal Probes

	clone	Gene	Total	Polysomal
5	13h04	Pyruvate kinase	No Change	Induced >>10
	5b08	Saposin	No Change	Induced >10
	9f11	Na,K-ATPase $\alpha$ -1 subunit	No Change	Induced x4
	1a04	Thymopoietin $\alpha$	No Change	Induced x4
	13h10	Poly(ADP-ribose) polymerase	No Change	Induced x5
	7c09	pM5	Reduced x2	Induced >6
	14e11	Ubiquitin	Induced x2	Induced x4
	10c06	Initiation Factor 4B	No Change	Induced x4
	1b09	90-kDa heat-shock protein	No Change	Induced >>10
	1c06	Acylamino acid-releasing enzyme	No Change	Induced >>10
	1e09	$\beta$ -spectrin	Reduced x2	Induced x5
10	3b04	Elongation factor-1-gamma	No Change	Induced x4
	13a12	Fibronectin	Induced x2	Induced x10
	7h12	Cytochrome C reductase core I	No Change	Induced >10
	9d12	Cytoskeletal $\gamma$ -actin	No Change	Induced >6
	13f09	Protein disulfide isomerase	Reduced x2	Induced >10
	9g12	DAP5		Induced x5

**TABLE 4**

### Statistics

<u>Probe</u>	<u>Number of differentials</u>	<u>Fold induction</u>
Total mRNA 4hrs HS	2	2
Polysomal RNA 1hr HS	14	2-4
	8	~8
	<u>15</u>	>10
	37	
Polysomal RNA 4hrs HS	13	2-4
	6	~10
	<u>18</u>	>10
	37	

TABLE 5

## Translationally controlled genes are identified by the 2A protease system

A. Ribosomal proteins or proteins directly involved in translation encoded by mRNAs containing 5' TOP#

S17 gbM13932  
S9 gb U14971  
EF-2 gbM19997  
L27a gb U14968  
L37a gbL06499

(Meyuhas et al., 1996)

B. Proteins encoded by mRNAs containing 5'TOP in their 5' UTR

Laminin binding receptor  
 $\beta$ 1-tubulin gb J00314

C. Gene with GC rich 5'UTR that regulates their translation

spermidine synthase gbM34338

retinol binding protein 5'UTR X00129

D. Unknown genes potentially regulated by translation

EST gb1059051	EST gb AA043162	EST gbW76915
EST gbT54424	EST gb AA025896	D45282
EST gbH15523	EST gb R07358	
EST gbW95821	EST gb H83477	
EST gbW99369	EST T34436	

E. Known genes that are potentially regulated by translation (and may contain IRES in their 5' UTR).

mitochondrial hinge protein gbS61826  
gp25L2 mitochondrial protein gp25L2  
mRNA encoding a protein related to lysyl t-RNA  
synthetase emb z31711  
SAP14 human spliceosome gb U41371

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20



## SEQUENCE LISTING

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  - (B) COMPUTER: IBM PC compatible
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  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "Primer"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATTATGCTG AGTGATATCT TTTTTTTTVV

30

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "Primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATTATGCTG AGTGATATCT TTTTTTTTAA

30

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATTATGCTG AGTGATATCT TTTTTTTTAC

30

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATTATGCTG AGTGATATCT TTTTTTTTATAG

30

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATTATGCTG AGTGATATCT TTTTTTTTCA

30

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATTATGCTG AGTGATATCT TTTTTTTTCC

30

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CATTATGCTG AGTGATATCT TTTTTTTTCG

30

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CATTATGCTG AGTGATATCT TTTTTTTTGA

30

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATTATGCTG AGTGATATCT TTTTTTTTGC

30

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CATTATGCTG AGTGATATCT TTTTTTTTGG

30

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATTAACCCTC ACTAAANNNN NNNNNN

26

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATTAACCCTC ACTAAATGCT GGGGA

25

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATTAACCCTC ACTAAATGCT GGAGG

25

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATTAACCCTC ACTAAATGCT GGTAG

25

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATTAACCCTC ACTAAATGCT GGTAG

25

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATTAACCCTC ACTAAGATC TGA CTG

26

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATTAACCCTC ACTAAATGCT GGGTG

25

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATTAACCCTC ACTAAATGCT GTATG

25

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATTAACCCTC ACTAAATGGA GCTGG

25

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATTAACCCTC ACTAAATGTG GCAGG

25

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATTAACCCTC ACTAAATGCA CCGTCC

26

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATCTGCGGT GA

12

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGCACTCTCC AGCCTCTCAC CGCA

24

## (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GATCTGTTCA TG

12

## (2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ACCGACGTCG ACTATCCATG AACA

24

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATCTTCCCT CG

12

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AGGCAACTGT GCTATCCGAG GGAA

24

## (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCAACTACCA TTTGGCCACT CAGGAAG

27

## (2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GCAACCAACC CTTCTCCACC AGCAG

25



## CLAIMS

What is claimed is:

1. A method for identifying translationally regulated genes, said  
5 method comprising the steps of:  
stimulating translation of an unknown target mRNA with a specific  
pathology or stress inducing element, the target mRNA being part of a larger  
sample of mRNA;  
dividing the sample of mRNA into pools of translated and  
10 untranslated mRNA; and  
differentially analyzing the pools of mRNA to identify genes  
translationally regulated by the stress inducing element.
2. A method as set forth in claim 1, wherein the stress inducing  
15 element is further defined as a stressor of unknown relationship to gene translation.
3. A method as set forth in claim 2, wherein the stress inducing  
element is a toxin.
- 20 4. A method as set forth in claim 2, wherein the stress inducing  
element is a chemical.
5. A method as set forth in claim 2, wherein the stress inducing  
element is a pharmaceutical.  
25
6. A method as set forth in claim 2, wherein the stress inducing  
element is an electric current.
7. A method as set forth in claim 2, wherein the stress inducing  
30 element is a pathogen.

8. A method as set forth in claim 2, wherein the stress inducing element is a pathological stress.

5           9. A method as set forth in claim 1, wherein at least two stress inducing elements are utilized to stimulate translation of separate aliquots of the target mRNA.

10           10. A method as set forth in claim 1, wherein said analyzing step is selected from the group consisting of differential display, representational differential analysis (RDA), suppressive subtraction hybridization (SSH), serial analysis of gene expression (SAGE), gene expression microarray (GEM), nucleic acid chip technology, direct sequencing and variations or combinations of these methods.

15           11. A method as set forth in claim 1, including the further step of cloning genes identified as being translationally regulated.

20           12. A method as set forth in claim 1, wherein said step of stimulating translation is further defined as chemically treating the cells.

            13. A method as set forth in claim 1, wherein said step of stimulating translation is further defined as irradiating the cells.

25           14. A method as set forth in claim 1, wherein said step of stimulating translation is further defined as depriving the cells of oxygen.

            15. A method as set forth in claim 1, wherein the cells are stimulated to differentiate.

30

16. A method as set forth in claim 1, wherein the mRNA sample includes cells that have undergone different treatments to stimulate mRNA translation in at least one pool of mRNA.

5                   17. A method as set forth in claim 1, wherein said analyzing step distinguishes between polysomal fractions that migrate in the same density on diffuse gradients or in a pool.

10                   18. A method as set forth in claim 1, wherein said analyzing step distinguishes between nonpolysomal fractions individually or as a pool.

15                   19. A method as set forth in claim 1, wherein said analyzing step distinguishes between stimulated polysomal and nonpolysomal fractions individually or in a pool.

20                   20. A method as set forth in claim 1, wherein said analyzing step distinguishes between each of the polysomal and nonpolysomal fractions individually or in a pool compared to an unfractionated total RNA pool.

25                   21. A method or process for identifying genes responsive to specific pathology or stress conditions including the steps of:

(a) applying a pathology or pathology-simulating stress to an organism or tissue or cells;

(b) isolating mRNA from the organism or tissue or cells subjected to the stress;

(c) dividing mRNA samples into at least two pools by its expression regulation and by its encoded protein localization; and

(d) differentially analyzing the pools of mRNA sample in comparison with control pools not subjected to the pathology or stress condition to identify genes that have responded to the pathology or stress condition.

22. The method according to claim 20, wherein said differential analysis is selected among differential display, representational differential analysis (RDA), suppressive subtraction hybridization (SSH), serial analysis of gene  
5 expression (SAGE), gene expression microarray (GEM), nucleic acid chip technology, direct sequencing and variations or combinations of these methods.

23. A method for identifying genes whose expression is regulated at the mRNA level under stress, said method comprising the steps of:

10 selectively stimulating translation of an unknown target mRNA with a stress inducing element, the target mRNA being part of a larger sample of mRNA;

dividing the sample of mRNA into pools of translated and untranslated mRNA; and

15 differentially analyzing the pools of mRNA to identify genes whose expression is regulated at the mRNA level by the stress inducing element.

24. A method as set forth in claims 1 or 23, wherein genes are identified at the translation level; genes regulated at the transcription level; genes  
20 regulated by RNA stability; genes regulated by mRNA transport rate between the nucleus and cytoplasm; and genes regulated by differential splicing.

25. A method as set forth in claim 23, wherein the stress inducing element is a toxin or a chemical, or a pharmaceutical or an electric current, or a  
25 pathogen or a pathological stress.

26. The method as set forth in claim 23, wherein said analyzing step is selected from the group consisting of differential display, representational differential analysis (RDA), suppressive subtraction hybridization (SSH), serial  
30 analysis of gene expression (SAGE), gene expression microarray (GEM), nucleic

acid chip technology, direct sequencing and variations or combinations of these methods.

5 27. The method as set forth in claim 24, wherein said step of stimulating translation is further defined as chemically treating the cells, or irradiating the cells, or depriving the cells of oxygen stimulated to differentiate.

28. A method for identifying gene sequences coding for internal ribosome entry sites, said method comprising the steps of:  
10 inhibiting 5'cap-dependant mRNA translation in a cell;  
collecting a pool of mRNA from the cells; and  
differentially analyzing the pool of mRNA to identify genes with sequences coding for internal ribosome entry sites.

15 29. A method as set forth in claim 28, wherein said inhibiting step is further defined as selecting for non-5'-cap dependent mRNA translation.

30. A method as set forth in claim 28, wherein said inhibiting step further includes the step of incorporating a gene coding for Polio virus 2A protease  
20 into the cell.

31. A method as set forth in claim 30, wherein said incorporation step is further defined as transforming the cell with a vector containing the gene coding for the Polio virus 2A protease.

25 32. A method as set forth in claim 30 including the step of controlling the expression of the gene coding for the Polio virus 2A protease.

30 33. A method as set forth in claim 28, wherein said analyzing step is further defined as differential display analysis.

34. A method as set forth in claim 28, wherein said analyzing step is further defined as representational difference analysis.

5 35. A method as set forth in claim 28, wherein said analyzing step is further defined as performing a gene expression microarray analysis.

36. A method as set forth in claim 28, including the further step of cloning genes identified as being translationally regulated.

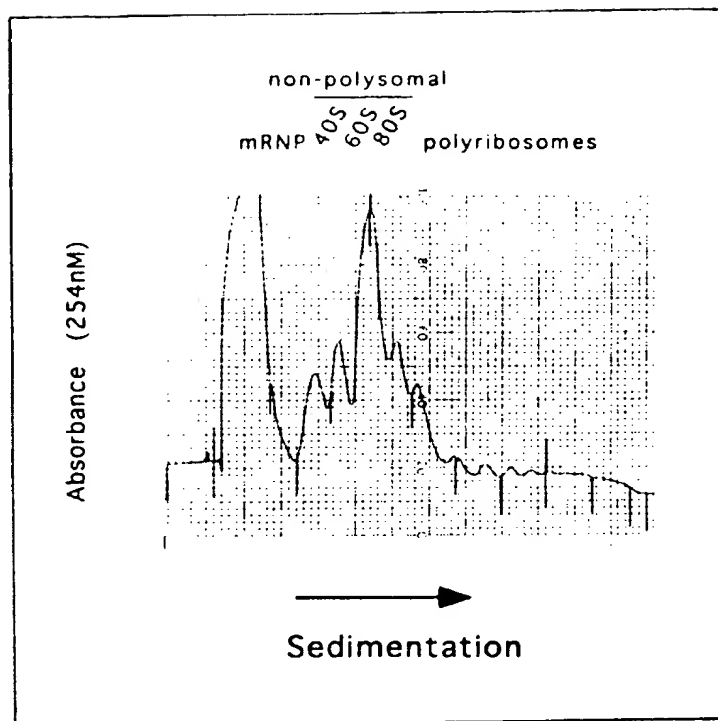
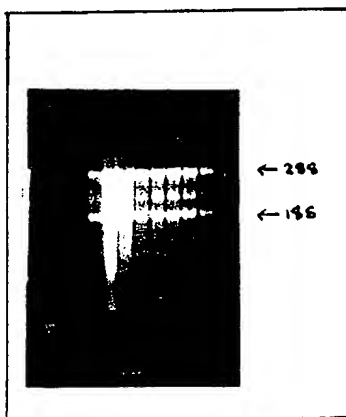
10 37. A method as set forth in claim 28, wherein said analyzing step distinguishes between polysomal fractions that migrate in the same density individually or in a pool.

15 38. A method as set forth in claim 28, wherein said analyzing step distinguishes between nonpolysomal fractions individually or as a pool.

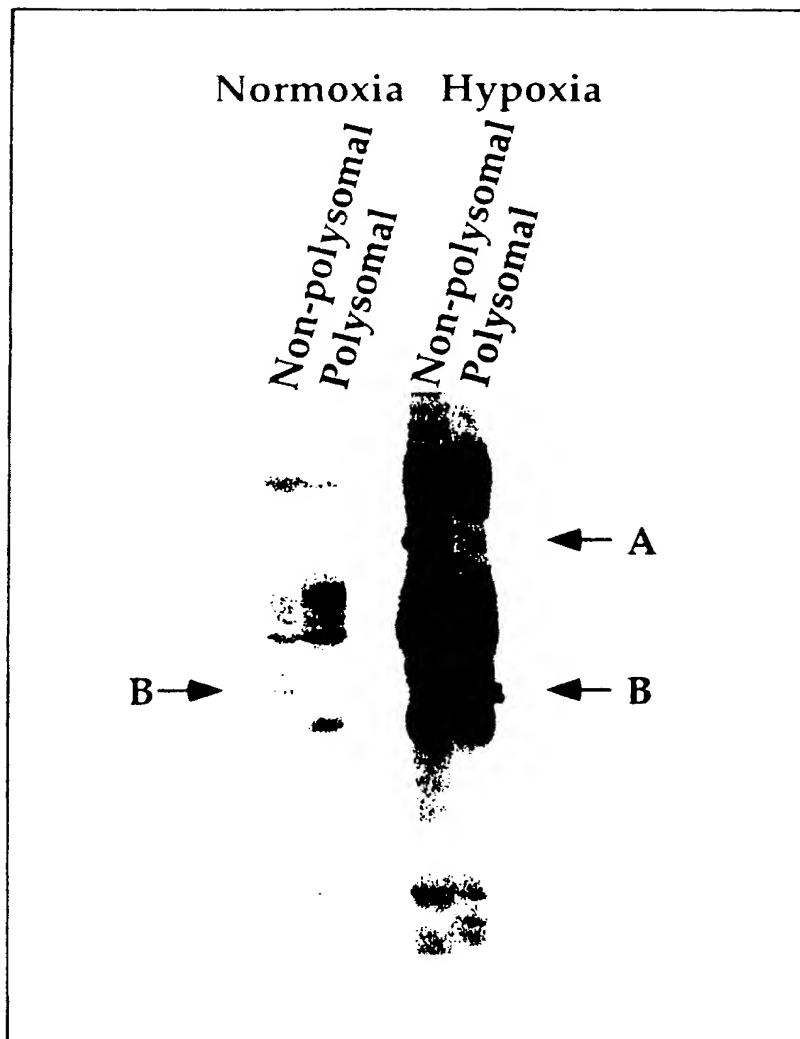
39. A method as set forth in claim 28, wherein said analyzing step distinguishes between stimulated polysomal and nonpolysomal fractions individually or in a pool.

20 40. A method as set forth in claim 28, wherein said analyzing step distinguishes between each of the polysomal and nonpolysomal fractions individually or in a pool compared to an unfractionated total RNA pool.

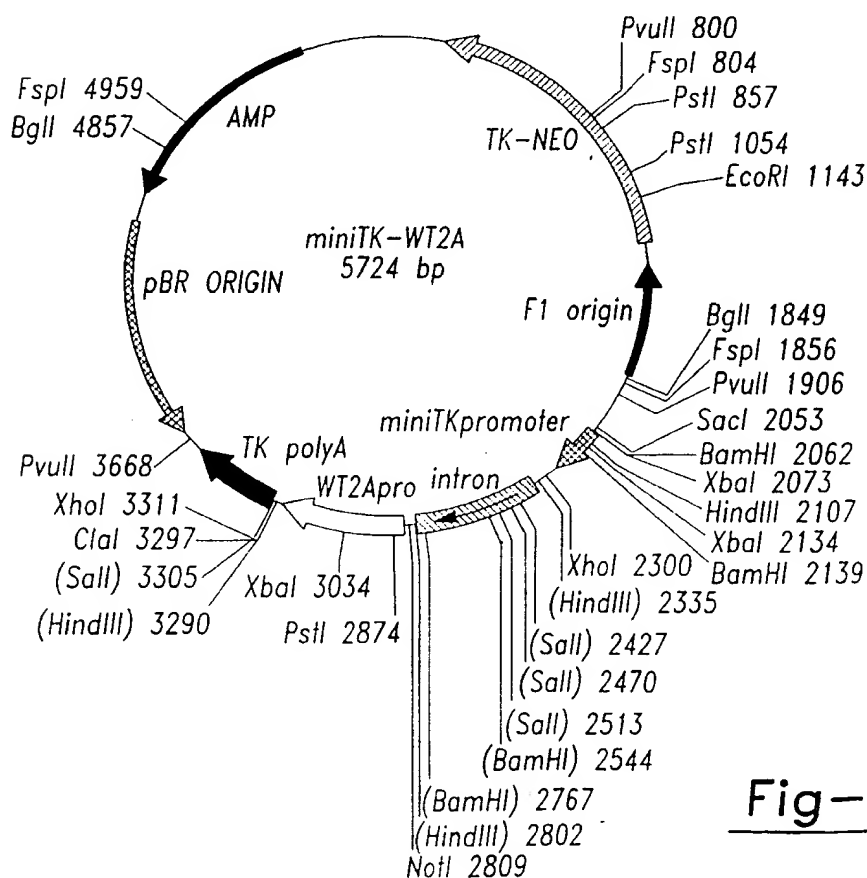
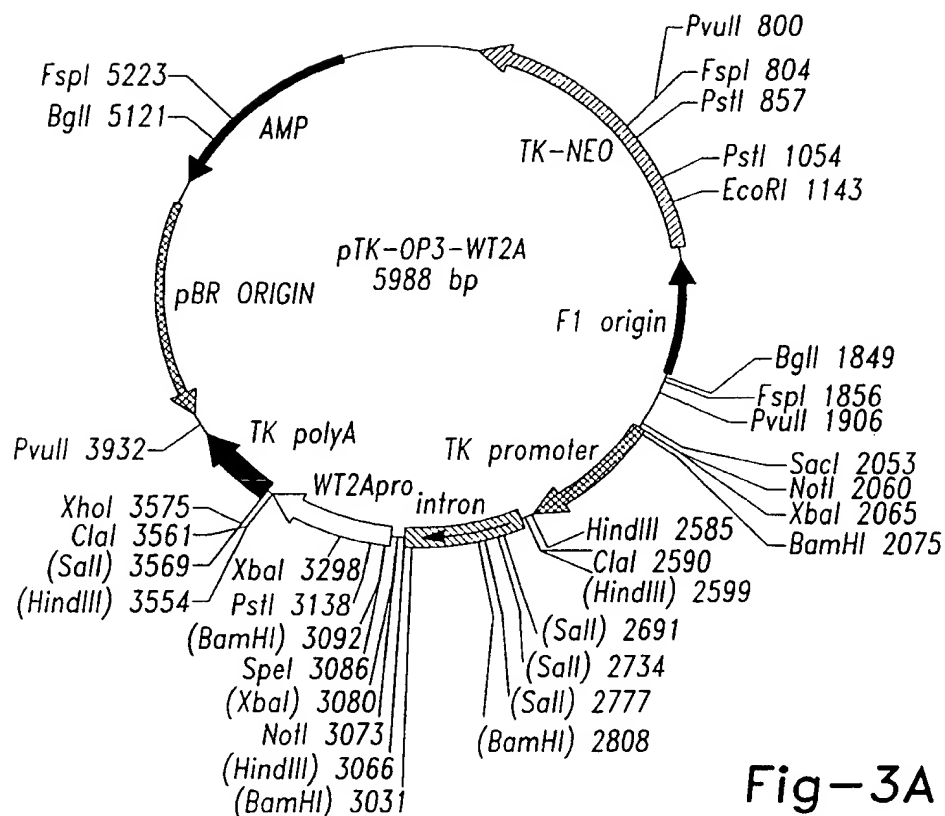
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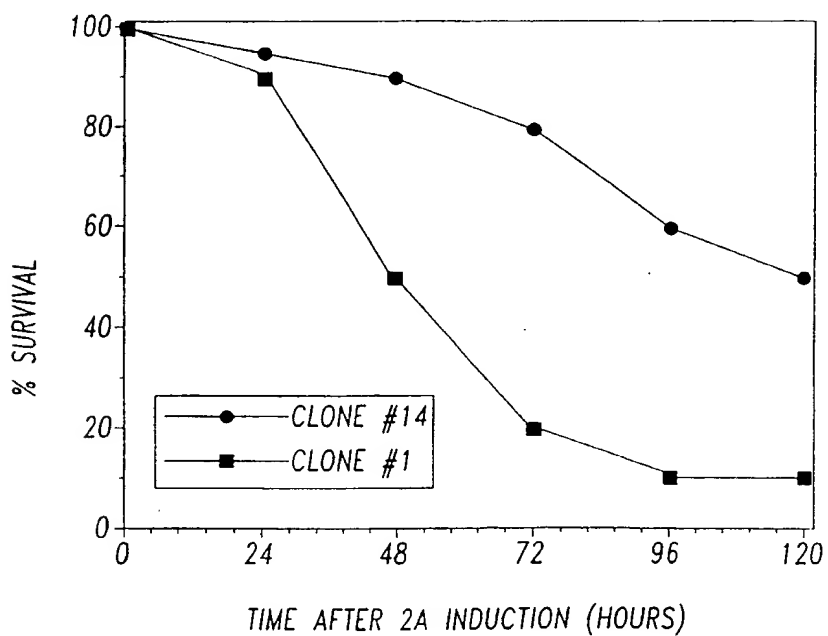
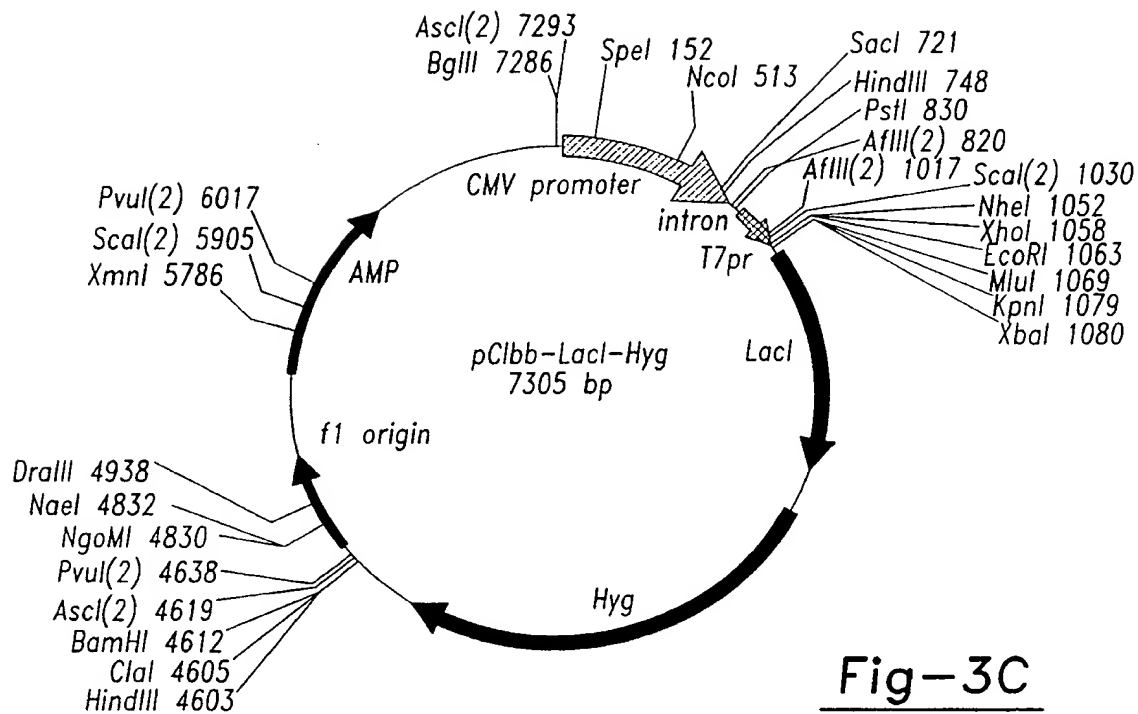
Fig-1AFig-1B

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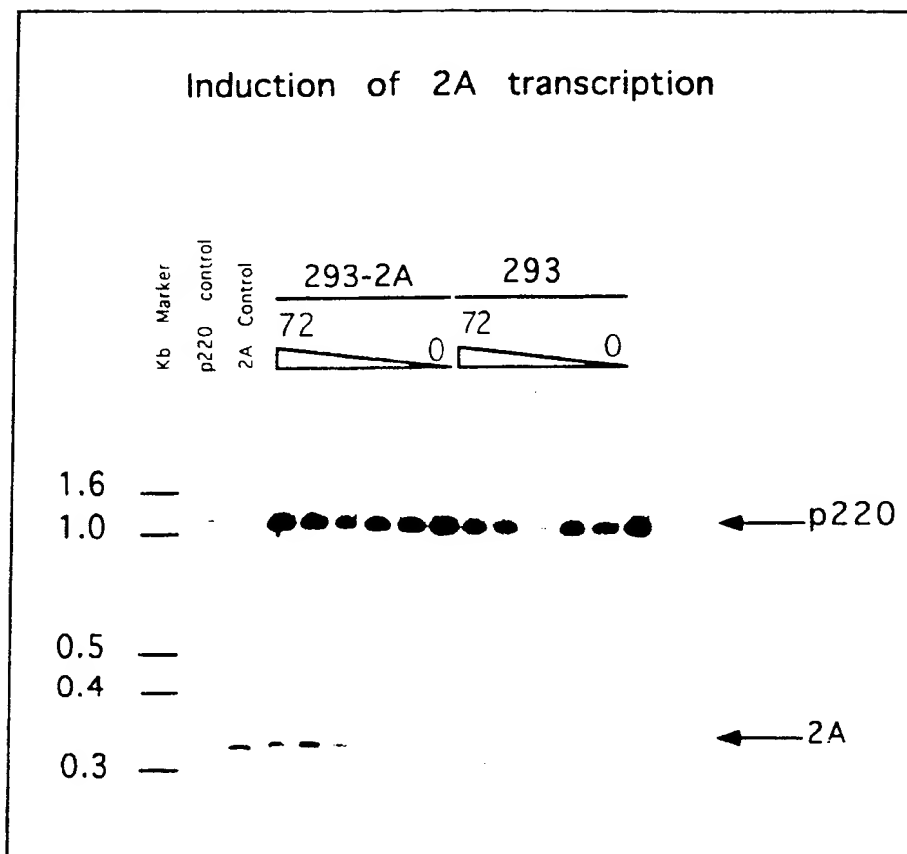
Fig-2





**Fig-4**

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*Fig-5*

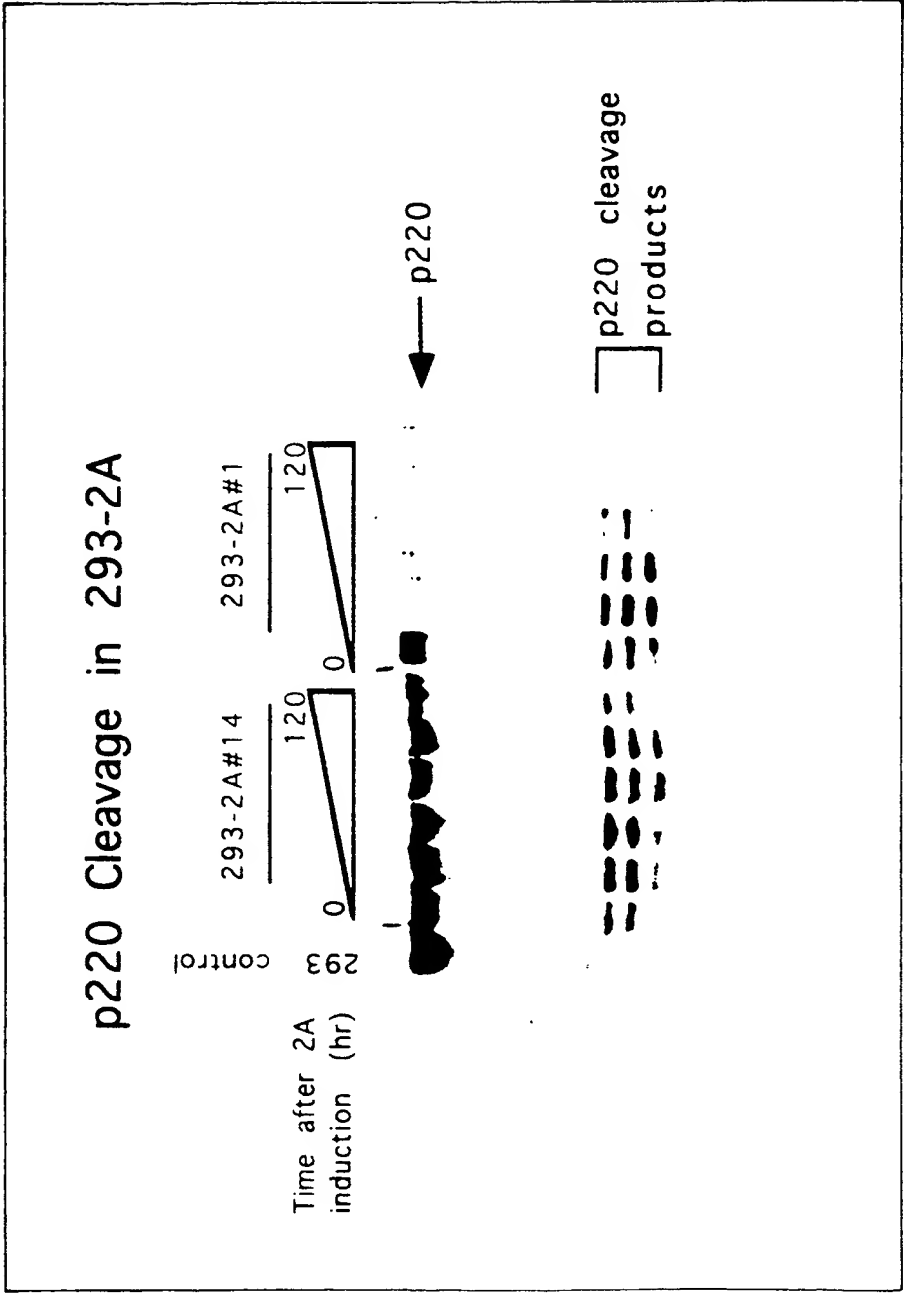


Fig-6

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/20831

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/10 C12Q1/68 C12N5/10 C12N9/50

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HANAUSKE-ABEL ET AL.: "DETECTION OF A SUB-SET OF POLYSOMAL mRNAs ASSOCIATED WITH MODULATION OF HYPUSINE FORMATION AT THE G1-S BOUNDARY; PROPOSAL OF A ROLE FOR eIF-5A IN ONSET OF DNA REPLICATION" FEBS LETTERS, vol. 366, 1995, pages 92-98, XP002059533 cited in the application	1-27
Y	see the whole document --- -/--	28-40



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

23 March 1998

Date of mailing of the international search report

06/04/1998

Name and mailing address of the ISA

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Authorized officer

Hagenmaier, S

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/20831

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WYCKOFF ET AL. : "EUKARYOTIC INITIATION FACTOR 3 IS REQUIRED FOR POLIOVIRUS 2A PROTEASE-INDUCED CLEAVAGE OF THE p220 COMPONENT OF EUKARYOTIC INITIATION FACTOR 4F" PNAS, vol. 87, 1990, pages 9529-9533, XP000371846 see the whole document ---	28-40
Y	THACH: "CAP RECAP: THE INVOLVEMENT OF eIF-4F IN REGULATING GENE EXPRESSION" CELL, vol. 68, 1992, pages 177-180, XP002059535 see the whole document ---	28-40
A	PELLETIER AND SONNENBERG: "INTERNAL INITIATION OF TRANSLATION OF EUKARYOTIC mRNA DIRECTED BY A SEQUENCE DERIVED FROM POLIOVIRUS" NATURE, vol. 334, 1988, pages 320-325, XP002059536 cited in the application See page 324, right column see the whole document ---	1-40
A	LINSKENS M H K ET AL: "CATALOGING ALTERED GENE EXPRESSION IN YOUNG AND SENESCENT CELLS USING ENHANCED DIFFERENTIAL DISPLAY" NUCLEIC ACIDS RESEARCH, vol. 23, no. 16, 1995, pages 3244-3251, XP002047039 cited in the application see the whole document ---	1-40
A	WO 95 13369 A (SCRIPPS RESEARCH INST) 18 May 1995 See page 31 see the whole document ---	1-40
A	LISITSYN N A: "REPRESENTATIONAL DIFFERENCE ANALYSIS: FINDING THE DIFFERENCES BETWEEN GENOMES" TRENDS IN GENETICS, vol. 11, no. 8, 1 August 1995, pages 303-307, XP000566341 see the whole document ---	1-40

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# INTERNATIONAL SEARCH REPORT

Inter Jnal Application No

PCT/US 97/20831

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KONDRATYEV A ET AL: "DETECTION OF GENES INDUCED BY IONIZING RADIATION IN HUMAN SQUAMOUS CARCINOMA CELLS BY DIFFERENTIAL DISPLAY OF MRNA"</p> <p>PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING 35 (0). 1994. 639,</p> <p>10 April 1994, XP002035451</p> <p>see the whole document</p> <p>-----</p>	13,27

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/20831

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9513369 A	18-05-95	US 5459037 A	17-10-95
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